Comparison of dietary sterols on growth, survival and midgut gland histology of prawn *Artemesia longinaris* (Decapoda, Penaeidea)

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Short running title: Sterols in diets of *Artemesia longinaris*

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Abstract

Two trials were conducted to evaluate the growth, survival and midgut gland histological conditions of Argentine prawn *Artemesia longinaris* nourished with diets having different sterols. Juvenile individuals of 0.97-1.01 and 1.75g were fed five semi purified diets containing 2% cholesterol, ergosterol, stigmasterol or β-sitosterol and a control ration without sterols for a period of six weeks. The digestive gland of intermoult prawn were dissected out and processed for light microscope studies. Rations having cholesterol and stigmasterol were found to result in higher growth than the other diets. Also individuals fed these diets showed midgut gland tissues similar to those of wild animals. The histoarchitecture of the midgutgland of *A. longinaris* fed ergosterol, β-sitosterol and the control diet were found to result in several alterations such as dismissing of cellular high, loss of the star–shaped lumen of the tubules, retraction of basal membranes and absence of brush border, haemocytic infiltration, cells with foamy appearance, cellular necrosis and hypertrophy and finally tissue disorganization. The results obtained suggest that addition of cholesterol or stigmasterol to feeds for this species, promotes an increase in weight and the hepatopancreas shows typical histological structure like wild prawns.

Introduction

The commercially valuable prawn, *Artemesia longinaris*, is distributed along the coastal waters of Argentina, Uruguay and Brazil from 22°30'S to 43°20'S (Boschi, 1963). The greatest abundance of this species occurs in Mar del Plata and Rawson, Argentina. Its annual catches are highly variable and therefore, it is important to determine the feasibility of culturing it at a commercial scale.

One of the main problems of growing penaeid shrimps is the inadequate knowledge of their nutritional requirements. Though nutritional principles are similar for all animals, the level of required nutrients in the feed varies with species. Feeding experiments had demonstrated the necessity of dietary sterols for growth and survival in prawn (Petriella et al. 1984). Sterols are considerate essential nutrients for crustacean since they are unable to synthesize these compounds and important components such as molting hormones, sex hormones, bile acids, and vitamin D are synthesized from cholesterol (Teshima and Kanazawa, 1971) Cholesterol is also a membrane component which is involved in the absorption and transport of fatty acids (Akiyama et al., 1991) and it is obtained directly from the diet or via the metabolic conversion of other dietary sterols (Kanazawa, 2001). Kanazawa et al. (1971) and Teshima and Kanazawa (1986) have indicated that some species of Crustacea possess the ability to dealkylate some C_{28} and C_{29} sterols such as sitosterol, ergosterol or stigmasterol via desmosterol to cholesterol.

The midgut gland (also known as digestive gland or hepatopancreas) is the largest organ by volume in decapods crustaceans and has many biological functions such as synthesis and secretion of digestive enzymes, absorption of digested products, maintenance of mineral reserves and organic substances, lipid and carbohydrate metabolism, distribution of stored reserves during the molt cycle and catabolism of some organic compounds (Ceccaldi, 1997). It is well known that a close correlation exists between the nutritional state and the histology of midgut gland (Vogt et al., 1985, Díaz et al., 2006). This gland is essentially composed of tubules lined by a simple epithelium with four cell types: E (embryonic), F (fibrillar), R (resorptive), B (blisterlike) and surrounded by connective tissue. E cells are situated at the end of the tubules and develop into: R cells involved in absorption, catabolism, storage of nutrients and detoxification of heavy metals, F cells synthesize digestive enzymes (Icely and Nott, 1992) and B cells have absorptive and degradative functions (Vogt, 1994, Johnston et al., 1998). In *Artemesia longinaris* is a bilobed brown organ which occupies much of the cephalothoracic cavity. It is connected to the pyloric stomach by two ducts (Petriella and Fonalleras, 1998). The organ is enclosed in a laminar connective tissue capsule and the morphological unit consists of a blind ending tubule with the four cellular types such as other crustaceans.

The aim of this work was to study the effects of different dietary sterols on growth and survival in *A. longinaris* and evaluate its effects on histological changes of the hepatopancreatic cells under culture conditions.

Materials and Methods

Two experiments of six weeks were conducted. The prawns, obtained from a commercial fisherman in the coastal waters of Mar del Plata, Argentina (38ºS) were maintained in 15 glass aquaria (150 l capacity) with marine water and filter beds of shell and sand.

Five isoproteic semi-purified diets were prepared employing the cold extrusion method (Fenucci, 1981) with the addition of 2% different sterols to a basal diet without sterols (Table 1) with a proximal composition of 44.5% protein, 8.7% lipids, 5.9% ash and 7.1% moisture.
Triplicate groups of 18 prawns/m^2 ranging 0.97-1.01 g (experiment 1) and 1.75 g (experiment 2) were stocked in each aquarium. Prawns were acclimated for seven days and daily fed *ad libitum* with the basal diet. The amount of food was adjusted according to their requirements during the feeding trials. Uneaten food, dead prawns and exuviae were daily removed before feeding. Also two groups of animals were maintained in total starvation only to evaluate the histology of the midgut gland in this severe condition.

Water temperature was 20±2ºC, salinity 33‰ and pH 7. Prawns were weighed at the beginning and at the finish of the experiments (44 days). At the end of the trials, individuals in intermolt stage were selected for histological studies; midgut glands were removed and fixed for 24 hours in Davidson´s fluid (Bell and Lightner, 1988), dehydrated in increasing concentrations of ethanol, clarified with benzene and finally embedded in paraffin. Tissue sections of 3 µm were stained with hematoxoylin-eosin, examined by light microscopy and photographed. Moult stage was determined by observation of the uropod setae development (Petriella, 1984).

Data were analyzed using Bartlett's test to determine homoscedasticity of variances, one-way analysis of variance (ANOVA) to determine significant differences among treatments, and chi square test for survival (Sokal and Rohlf, 1995). Limits of significance for all critical ranges were set at $p<0.05$

Results

For both feeding trials, mean weight, percentage of weight gain and survival of prawns fed semipurified diets containing different sterols and the control diet are presented in Table 2. Final mean weight and the percentage of weight gain of individuals nourished ergosterol diet were not included because they exhibited very low survival. Statistical differences in survival were only found between animals nourished ergosterol diet and the others.

At the end of first experiment, final mean weight and percentage of weight gain of prawn fed diets containing cholesterol (1.89g; 89%) and stigmasterol (1.87g; 87%) were statistical higher than the control without sterols (1.55g; 55%) whereas no differences were observed among the values of prawn fed rations containing β-sitosterol (1.65g; 69%) and all the treatments.

Coincidently in the second experiment, final mean weight and percentage of weight gain of prawn fed cholesterol (3.48g; 99%) and stigmasterol diets (3.42g; 96%) were significantly higher than the control treatment (2.74g; 57%) moreover no differences were observed among values of β-sitosterol feed (3.05g; 75%) and every other.

Wild prawn midgut gland is composed of many tubules; like other crustaceans four kinds of cells form a lined tubules epithelium named cells E, R, F and B (Petriella and Fonalleras, 1998). Figures 1 and 2 show transverse sections of the tubules through the middle region of animals fed stigmasterol and cholesterol diets respectively. They exhibited cell structure and star-shaped lumina similar of those of wild prawn. Brush border is marked on the luminal surface of the cells. A myo-epithelial layer surrounds the tubules. Haemocytes are scarce and confined to the haemal spaces between tubules.

Cytology of the midgut gland of individuals fed β-sitosterol diets revealed haemocytes infiltration, cellular retraction, desquamation and wavy basal membranes. Many tubular lumina lost their star like form and the brush border (Figure 3).

Tubules are separated due to cellular retraction in midgut gland of prawn nourished ergosterol diet; vacuolization became more prominent and the cells took a foamy appearance (Figure 4). Individuals fed a free sterols diet evidenced with hypertrophy and shrinkage of cells and ample tubular lumina (Figure 5); some areas revealed haemocytic encapsulation and severe necrotic focus (Figure 6).

After 14 days starvation cell vacuolization became more prominent and certain parts of midgut gland animals showed lost the typical acinar structure of hepatopancreatic tubules and disorganized tissues (Figure 7).

Discussion

Most animals are capable of synthesize sterols from acetate, but crustaceans are incapable of doing that (Teshima and Kanazawa, 1971). Several experiments revealed that crustaceans need an outer source of sterols (Martinez Romero et al. 1991, Haran and Fenucci, 1996). Cholesterol may be a constituent of membranes and a precursor of steroid hormones, moulting hormones and cholesteryl

esters in penaeid shrimps. Kanazawa (2001) reported required levels of dietary cholesterol from 0.1 to 2.0% of the dry weight of diets. Martinez Romero *et al*. (1991) found an optimum level of digestibility for 2.3% of dietary cholesterol in *Artemesia longinaris* and they indicated the probable existence of a saturable carrier system at the level of the epithelia where cholesterol is absorbed; whereas 0.5-2.0% resulted optimal for survival.

Sterol composition of crustacean corresponds to 90-95% of cholesterol (Kanazawa, 2001). After 30 days, total sterol contented in the body tissues of *Marsupenaeus japonicus* decreased in prawn fed a sterol-free diet but individuals fed ergosterol, stigmasterol or β-sitosterol diets, had a content of sterol similar to the prawn before the feeding trial (Kanazawa *et al.,* 1971). These results suggest that cholesterol and other sterols were absorbed and utilized. Due to the fact the relative percentage of cholesterol was found to be high and no detectable increase of the others was perceived, authors suggested that *M. japonicus* convert these C_{28} and C_{29} sterols to cholesterol. Besides ergosterol, stigmasterol and β-sitosterol were effective on the growth rate but lower than cholesterol. In this study, during six weeks, diets containing cholesterol, stigmasterol or β-sitosterol were found to sustain growth of *A. longinaris* in both experiments; although initial mean weight was different in each, the patterns of growth were similar.

Bioconversion rates and the consequent nutritional value of sterols other than cholesterol in Crustacea may be species specific (D´Abramo et al., 1984). The effective utilization of some dietary phytosterols apparently reflects the feeding habits of omnivorous species compared with an apparently exclusive requirement for cholesterol exhibited by crustacean carnivorous species (D´Abramo and Daniels, 1994). Total replacement of cholesterol with a mixture of phytosterols composed primarily of β-sitosterol did not yield good growth and survival in juvenile lobsters *Homarus sp.* (D´Abramo *et al.,* 1984). On the other hand a mixture of phytosterols is as valuable as cholesterol in satisfying the dietary sterol requirement of juvenile *Macrobrachium rosenbergii*. The present study revealed that cholesterol stigmasterol and stigmasterol diets have benefit effects in the growth but did not affect survival *in Artemesia longinaris,* an omnivorous-carnivorous species.

Changes in the histology of the hepatopancreas can be observed before other body responses (Vogt et al., 1985, Esteve and Herrera, 2000). Midgut gland cytology of *A. longinaris* maintained at 16‰ salinity revealed epithelial necrosis, haemocytic nodules and epithelial desquamation due to alterations in environmental conditions (Masson, 2001). Besides the growth results obtained with the different experimental diets used are related to histological characteristics because previous studies demonstrated that cells and tissues of the midgut glands of Crustacea are very sensitive to diverse ingredients in diets. Díaz et al., (2002) analyzed the hepatopancreas structure of *Palaemonetes argentinus* provided for different levels of dietary cholesterol; Kumaraguru Vasagam *et al*., (2007) related dissimilar treatment process on antinutritional factors present in grain legumes to histologycal anormalies in hepatopancreas of *Penaeus monodon*. Variations of the cytological characteristics of the midgut gland induced by sub optimal levels of dietary vitamin E and A have been reported for *Pleoticus muelleri* and *Artemesia longinaris* by Fernández Gimenez (2002). Also marked histological alterations were evident in cells of young *A. longinaris* fed diets deficient in methionine and levels of methionine higher than 10%; the most notable changes observed were shrinkage of cells and microvilli as well as condensation of nuclear chromatin, cellular hypertrophy, cellular desquamation towards the tubular lumen, haemocytic infiltration, nodules or tumor structures, with general collapse of the tubules and cellular necrosis (Romanos Mangialardo, 2006). In this study individuals of *A. longinaris* fed on β-sitosterol and ergosterol diets showed some alterations, infiltration of haemocytes, cellular retraction and cells with foamy appearance. Focus of encapsulation, vacuolization and cellular retraction and desquamation were observed in individuals fed diets without sterols whereas hepatopancreas of animals on extreme condition such as 14 days starvation showed tubular disruption, the most serious alteration.

Under the experimental conditions, the results indicate that diets containing cholesterol or stigmasterol showed good results for growth of prawn and coincidently its hepatopancreas exhibited similar histology described for wild individual.

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Table 1. Composition (g/100g diet) of experimental semi purified diets.

	Diets				
	Cholesterol	**Ergosterol**	**β-sitosterol**	**Stigmasterol**	**Control**
Sterols	2.0	2.0	2.0	2.0	-
Cellulose	10.0	10.0	10.0	10.0	12.0
Casein			35.5		
Squid protein [a]			3.0		
Corn starch			22.0		
Gelatin			12.5		
Sodium alginate			3.7		
Hexametafosfato			1.0		
Soy lecithin			1.0		
Vitamin mix [b]			2.0		
Fatty Acids [c]			7.0		
Minerals [d]			0.3		

[a] Díaz *et al* (1999)

[b] Vitamin mix (mg/kg): thiamin 163;rivoflavin 156; pyridoxin 213; cianocobalamin 20; biotin 250; folic acid 25; niacin 250; inositol 300; ascorbic acid Rovimix STAY C (Sigma) 781; cholecalciferol 35;menadione 34; vitamin A acetate 100; calcium pantothenate 250; choline cloride 300; vitamin E (Laboquímica Argentina) 1500.
[d] calcium 1,000 mg; magnesium 500 mg; potasium 99 mg; zinc 30 mg; iron 10 mg; copper 2 mg; iodine 150 µg; selenium 200 µg; molybdenum 500 µg (Twin Laboratories, Inc. USA).
[c] Polinsaturated fatty acids ω-3 PUFAs. (Omega Sur, Mar del Plata, Argentina).

Table 2. Mean weight, weight gain and survival percentage of *Artemesia longinaris* fed diets containing cholesterol, stigmasterol, β sitosterol, ergosterol and the control diet without sterols

DIET	1st experiment				2nd experiment			
	Mean weight (g)		Weight gain (%)	Survival (%)	Mean weight (g)		Weight gain (%)	Survival (%)
	Initial	Final			Initial	Final		
Cholesterol	1.01±0.223	1.89±0.415^{a}	89.20^{c}	66.66^{e}	1.75±0.290	3.48±0.561^{g}	99.29 ^{j}	74.00^{l}
Stigmasterol	1.00±0.203	1.87±0.375^{a}	86.50^{c}	76.66^{e}	1.75±0.329	3.42±0.622^{g}	96.06 ^{j}	74.00^{l}
β sitosterol	0.97±0.214	1.65±0.409^{ab}	69.27^{cd}	70.00^{e}	1.75±0.325	3.05±0.528^{gh}	74.77 ^{lk}	74.00^{l}
Ergosterol	0.99±0.207	---	---	16.66^{f}	1.75±0.367	----	----	29.63^{m}
Control	1.00±0.195	1.55±0.292^{b}	55.07 ^{d}	76.66^{e}	1.75±0.314	2.74±0.227^{h}	56.95 ^{k}	74.00^{l}

Different superscripts in a column indicate significant differences

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Figures 1-7. Transverse sections of midgut gland tubules through the middle region of prawn *Artemesia longinaris* fed semi purified diets containing or not sterols and total starvation. Bar 25 μ

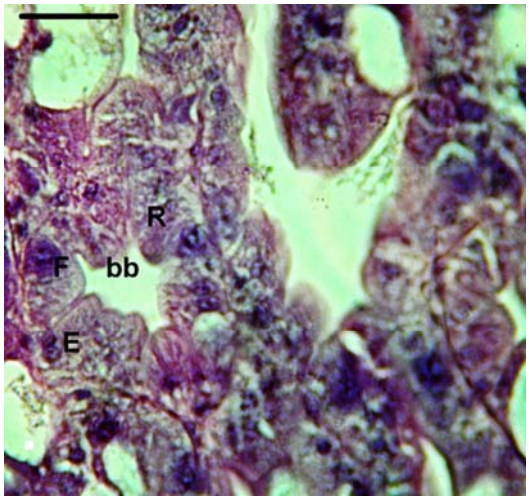

Figure 1. Prawn fed stigmasterol diet. bb: brush border; E: E cel; F: F cel; R: R cel; L: lumen

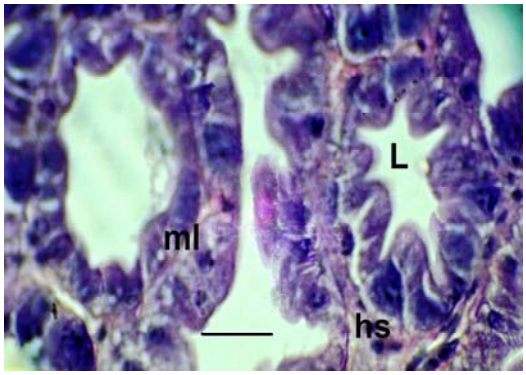

Figure 2. Prawn fed colesterol diet. hs: haemal sinus; L: lumen; ml: myo-epithelial layer

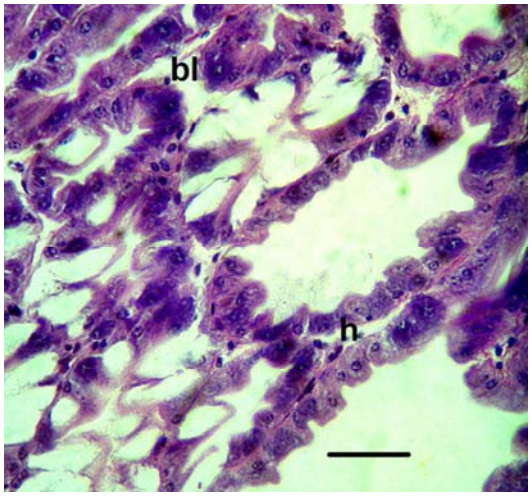

Figure 3. Prawn fed β-sitosterol diet. h: haemocytes; ml: wavy basal membranes

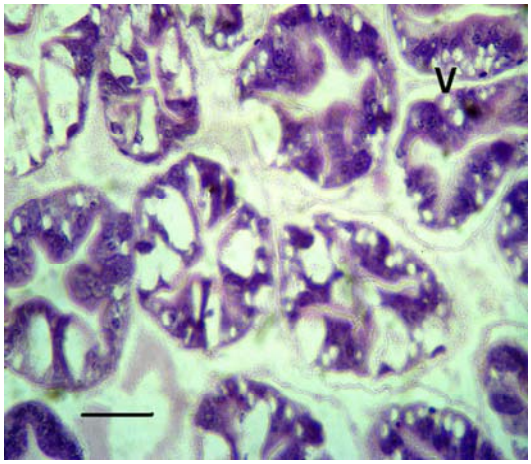

Figure 4. Prawn fed ergosterol diet. V: vacuoles. Note tubular separation and foamy cells.

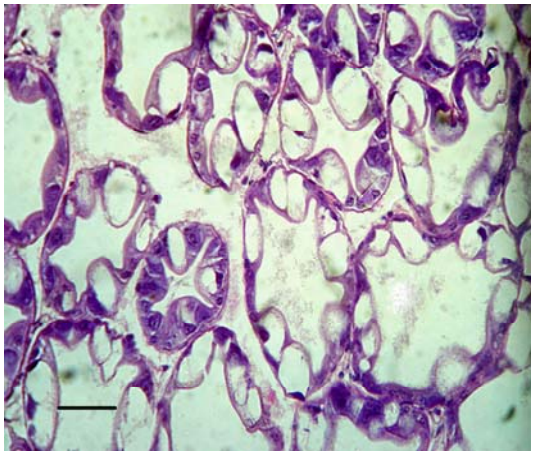

Figure 5. Prawn fed free sterols diet. Note hypertrophy of B cells and large tubular lumina.

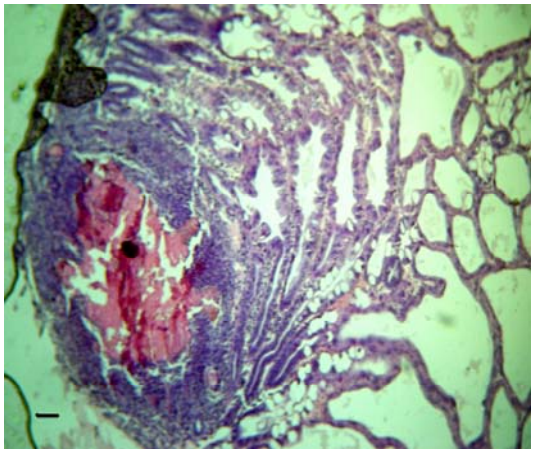

Figure 6. Prawn fed free sterols diet. Note necrotic focus.

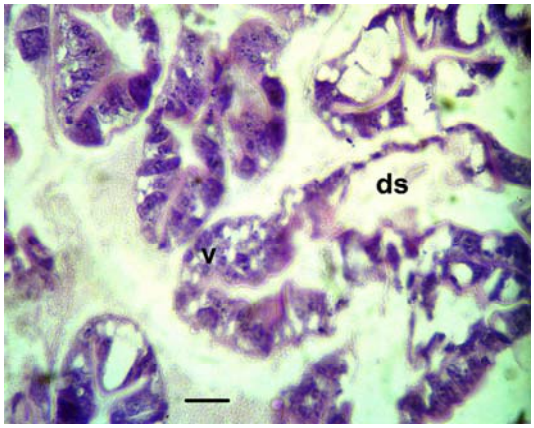

Figure 7. Prawn in 14 days starvation. Note tubular separation, cell vacuolization and tubular disorder.